Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

CARCINOGENESIS

No. 896

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

RECEIVED
JAN 18 1973

Application For Research Grant

Date: Jan. 12, 1973

1. Name of Investigator(s): (include Title and Degrees)
Joel Warren, Ph. D., Director, Leo Goodwin Institute for Cancer Research
Miriam R. Sacksteder, B. S., Research Associate

2. Institution & Address:
Leo Goodwin Institute for Cancer Research
3301 College Avenue
Fort Lauderdale, Florida 33314

3. Short Title of Project:
Potentiation of Herpesvirus by Tobacco Tar Condensates

4. Proposed Starting Date:
As soon as possible

5. Anticipated Duration of this Specific Study:
One Year with option of extension

6. Brief Descripton of Objectives or Specific Aims:

This proposal is one of a group of studies into the possible role of HVH-1 in human pulmonary cancer. Concurrent research includes: 1) a study of HVH-1 antibody levels in the sera of normal and cancer patients and their relation (if any) to smoking history; 2) attempts to isolate HVH-1 from normal and neoplastic human lung with the aid of DMSO-BUDR; 3) demonstration of HVH-1 fluorescent antigen in normal and neoplastic pulmonary tissue.

Specific aims of the proposal:

A. We will establish cultures of several cell lines from normal human bronchial and alveolar tissues. These will be maintained as monolayers by established procedures.

B. We will investigate the multiplication of HVH-1 virus in such cultures and whether the presence of tobacco tar condensates in the medium modifies its infectivity.

C. We shall expose human lung cells to tobacco tars in vitro and observe them for evidence of transformation in systems containing live or inactivated HVH-1.

D. The activity of tars known to be carcinogenic in mice and those which promote RSV and MSV will be compared with inactive tars for their effects on human lung tissue and herpesvirus infection.

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7. Give a Brief Statement of your Working Hypothesis:
See attached.

Source: https://www.industrydocuments.ucsf.edu/docs/tyyl0000

8. *Details of Experimental Design and Procedures:* (Attach Separate Pages)

See attached.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

See attached.

10. Additional Requirements:

None.

Biographical sketches of all principal and professional personnel (append)

See attached.

12. List of publications: (Five most recent as pertinent) (append)

See attached.

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13. Budget: (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
Joel Warren, Ph. D., Principal Investigator	10%	
Miriam Sacksteder, B. S., Co-Investigator	30%	
Fringe Benefits @ 13%		REDACTED
Technical		
Harriet Jarosz, B. A.	100%	REDACTED
Fringe Benefits @ 13%		
Sub-Total		$14,916
B. Consumable Supplies (list by categories)		
Tissue culture media, chemicals, sera		2,200
Glassware		350
Photographic and histology reagents		150
Sub-Total		$ 2,700
C. Other Expenses (itemize)		
Hospital travel for specimens		275
Histology services		500
Photographic and graphic supplies		300
Sub-Total		$ 1,075
D. Permanent Equipment (itemize)		
Microscope, binocular, inverted		1,500
		$ 1,500
E. Overhead (15% of A+B+C)		$ 2,803
Total		$22,994

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Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	Cannot be established at this time					
Year 3						

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature [illegible]
Director of Project and Institute 587-6660
Telephone

Signature J. C. Reinke
[redacted] of the Institution
Administrative Assistant "
Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Research Involving Employment of Sarcoma Virus to Develop a Short-Term Bioassay System and to Evaluate the Biological Activity of Smoke Condensate	U. S. Department of Agriculture	$10,800	6/24/71 - 6/23/72
The Role of Herpesvirus hominis, Type 1, in Human Pulmonary Cancer	Damon Runyon Foundation	$16,000	10/1/72 - 10/31/72
Student Stipend for E. M. Twist	Nova University	R	Annual
General Support, Laboratory Supplies	Leo Goodwin Institute	$ 1,000	Annual

ending

Detection and Isolation of Herpesvirus from Normal and Neoplastic Human Pulmonary Tissues	National Institutes of Health	$20,789	One year.

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7. Working Hypothesis

If one assumes that a viral oncogene is involved in the initiation of human primary lung cancer, then a series of related conclusions follow. The virus (s) should be ubiquitous and persist even in the presence of antibody. Frequent access to the bronchial tree and alveolar membranes from an oropharyngeal habitat would be likely. The agent should belong to a family having oncogenic "relatives" and should be oncogenic and/or teratogenic in experimental systems.

Herpesvirus possesses all of these attributes and has established a commensal relationship with the entire human race and probably all of the higher primates. Approximately 2-3% of asymptomatic adults carry the agent in their oropharynx, and the latent and recurrent characteristics of clinical infection are well known (1). It is estimated that overt illness occurs in only 10-15% of primary infections (2). Clinical recrudescence from the carrier state can be precipitated by a wide variety of physical and chemical stimuli, including trauma (3). Within the past four years, the existence of at least two strains of HVH has been well documented (4). These correlate with the site of virus isolation: Type 1 from non-genital, mainly oral sites, and Type 2 almost exclusively from genital tissues. Transmission of Type 2 appears to be venereal, and its oncogenic role in carcinoma of the cervix is being increasingly substantiated (5,6). The viruses of Burkitt lymphoma, Marek's disease, a primate leukemia and frog kidney cancer, similar to HVH in size and structure, are oncogenic relatives of the herpes group.

Evidence that HVH-1 specifically can be oncogenic is only fragmentary at present. Certain oral strains, "HF" and "JES", induced chromosomal aberrations in tissue culture (7,8). Tanaka and Southam reported that Type 1 strain enhanced development of methylcholanthrene-induced carcinomas in mouse skin (9). We have recently confirmed this (10). HVH was the only virus of several tested found to react with antisera to HeLa cells in vitro (11). Further suggestion of the oncogenic potential of Type 1 strains lies in observations of its teratogenicity in hamsters (12) and the chick embryo (13).

Three of the herpesviruses, MDHV, EBV and HVH are known to exist in a proliferating, stimulatory, non-permissive, and often oncogenic, association with lymphoid cells. HVH can do this in non-natural hosts and characteristically destroys infected cells in vitro soon after infection. Yet the chronic persistence of HVH in man (possibly in sensory ganglia) suggests that it exists in a state of low or non-infectivity under certain conditions.

The potential of non-replicating herpesvirus to be oncogenic has recently been demonstrated by Rapp and his associates (14), who found that hamster embryo fibroblasts underwent malignant transformation when exposed to HVH-2 irradiated by ultraviolet light. The continual involvement of the HSV-2 genome in the neoplasm was demonstrated by immunofluorescence

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and neutralizing antibody in the sera of tumor-bearing hamsters. This resembles the persistence of EBV antigen in cells of Burkitt's lymphoma (15). Whether the persistence of viral genome is required for oncogenicity is not known. Nevertheless, we believe it important to establish whether persistent exposure to low concentrations of tobacco tar, as in the lungs of smokers, could activate latent or infectious herpesvirus (and possibly be an immunosuppressant as well (16) resulting in malignant transformations in the pulmonary tree.

There is considerable literature describing the effect of tobacco smoke or tars on the cell and its nucleic acid in vitro. (For a general review, see Larson and Silvette, ref. 17). However, these studies have been performed primarily with rodent pulmonary tissues. When virus-tobacco smoke conbinations were employed, these were investigated in the intact animal (18). The observation that the addition of tobacco "tars" to lung explants induced chromosomal alteration and changes in nuclear DNA (19) encourages us to expect changes in the replication patterns of a DNA virus in the presence of tar. We can find no references to the deliberate combination of HVH and tobacco tar in the same cell system in vitro or in animals although Goldberg, Docherty and Rapp (20) recently demonstrated that 1,2-dimethylbenzanthracene (DMBA) inhibited HVH-2 replication in rabbit kidney cell cultures by blocking synthesis of the viral DNA. Since tobacco tar also contains a variety of polycyclic aromatic hydrocarbons, one could conceive of tar suppressing the cytotoxic effects of HVH-1 in the lungs and yet permitting expression of oncogenicity. Tars will also enhance viral infection and, during the past two years, we have demonstrated that certain tobacco tar condensates are capable of promoting infection by an oncogenic RNA virus, RSV in quail (21). The effect is reproducible with a given tar and related to its composition and mode of tobacco extraction. Freeman, et al., have shown that similar tars will promote the transforming effect of the MSV-0 strain of Moloney sarcoma virus in rat fibroblasts (22). In the light of the preceeding discussion, we propose to investigate the question: Can tobacco tar condensates promote or convert HVH-1 into an oncogenic process in lung tissue in vitro?

8a. Procedures

Viruses

Two oral strains of herpesvirus will be used: a recent human isolate, the HSV-1 line received from Dr. B. Roizman, and the older, classical HF strain obtained from Dr. C. Southam. Both strains are currently used in our laboratory for other research. They are maintained in BALB/c mice, Fischer 344 rats, CETC and HEP-2 cell cultures. Standardized virus pools retain stable endpoints at -70° for at least four months when titrated by plaque assay.

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Tobacco Tars

Through the collaboration of Drs. F. Bock (Roswell Park Memorial Institute) and T. C. Tso (U. S. Department of Agriculture), we have obtained 32 experimental cigarette smoke condensates made from experimental flue-cured tobaccos of four entries, each with eight stalk positions. These have been frozen and, when needed, are diluted to a 0.1% solution in DMSO before addition to tissue culture. These same fractions are being tested by Dr. Bock for carcinogenic activity in the mouse skin assay, and his findings could eventually be correlated with our own.

Tissue Culture

Normal, human, fetal and adult, pulmonary and bronchial tissues will be obtained through the collaboration of Dr. R. Poppiti, Department of Pathology, Broward General Medical Center. These will be selected from biopsy and surgical specimens taken from cases of non-malignant disease. The cultivation of human lung tissues has been well described by Sherwin, Richter and Richter (23) and Wellings and Jentoft (24), and the methods proposed are based on their work.

Small (±0.5 cm or less) tissue fragments will be collected in chilled Eagle's minimal essential medium with double strength antibiotics. Within one hour after transport to the laboratory, they will be repeatedly washed in the above and 1-3 mm explants then placed on a cellophane raft in an organ culture dish in double strength MEM, glucose increased to 130 mg/ml, and 30% fetal bovine serum. When explants are established, they will be minced, trypsinized, and monolayer preparations initiated in Leighton tubes in order to obtain relatively "clean" cultures of epithelial cells. Depending upon results, we plan to select 2 or 3 of the most flourishing lines for the work described below. Because fetal bovine serum has been reported to inhibit spontaneous neoplastic transformation in vitro (25), some culture replicates will be fed with 10% filtered, pooled, human serum obtained from the blood bank of Broward General Medical Center. Since lung tissue monolayers are relatively short lived, (2-6 weeks), we shall periodically go back to explant cultures to renew the monolayer lines.

8b. Experimental Design

Effect of Prolonged Exposure to Tobacco Tars on Transformation of Human Pulmonary Epithelium

Cultures derived from bronchial and alveolar tissues will be serially propagated for several weeks in EMEM containing 0.1 mcg. of different tobacco tar condensates. The latter will be selected on the basis of their plant topography and activity in the mouse skin and sarcoma virus assays. Treated and control cultures will be periodically observed for the following criteria of transformation:

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1) Growth of the cells in semi-solid agar.
2) Changes in colonial and cell morphology as revealed in Giemsa-stained films and by electron microscopy.
3) Changes in the chromosome karyotype.
4) Capacity to multiply in the cheek pouch of the hamster (Graffi line) and in the sub-cutis of the irradiated Fischer 344 rat. The rodents will be sacrificed at appropriate times and the fate of the inocula determined by histological examination.

Effect of Tobacco Tars on the Infectivity of HVH-1

Monolayer cultures of human and pulmonary epithelial cells will be infected with graded doses, 1-1000TCID50%, of both strains of HVH-1 virus in a methylcellulose overlay plaque assay using two plates for each dilution. A similar series of titrations will be performed in cultures containing 0.1mcg and 1.0 mcg/ml of a tobacco tar condensate. Controls will include non-infected cells and cultures with added tar alone. Four days after infection, a 0.005% solution of neutral red will be added to facilitate plaque counting. This type of experiment will be repeated with each of the 32 available tars. Fluorescent antibody staining for HVH-1 antigen will also be applied to these cultures.

In order to determine whether prior exposure to tobacco tar modifies the response to HVH-1, a series of lung cultures will be exposed to tolerated concentrations of tobacco tars for four days, following which the cells will be transferred to fresh, tar-free media and immediately used for titration of HVH-1. Both cytopathic end points and cell morphology will be compared in treated and control plates.

Will Inactivated HVH-1 Transform Human Lung Tissue and Can This Be Potentiated by Tobacco Tar?

As suggested by the work of Duff and Rapp with hamster embryonic fibroblasts and HVH-2 (14), we shall expose HVH-1 as a 0.5 cm film to a GE8T5 ultraviolet lamp for varying intervals to establish its inactivation curve. Following this, we shall prepare irradiated, non-infectious and low infective pools of virus and determine whether the presence of tobacco tar condensates potentiates the ability of HVH-1 to transform human cell cultures. The occurrence or persistence of fluorescent HVH-1 antigen in such cultures would provide an important lead for further exploration. These will also be examined by electron microscopy for the presence of "C" type particles.

Significance of this Research

Prolonged exposure to tobacco tars is a significant pre-disposing factor in the susceptibility to lung cancer. The demonstration of a co-carcinogenic effect of some or all tars on viral inhabitants of the human pulmonary tract could open an important experimental approach to defining the mode of action of tobacco tars as well as explaining the pathogenesis of human pulmonary neoplasia.

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9. Facilities

Nova University, chartered in 1964, is an independent, non-sectarian and non-profit institution for graduate study and research in science and technology and has been accredited by the Southern Association of Schools and Colleges.

The Life Sciences Center, established in 1969, occupies approximately 26,000 sq. ft. in the Louis W. Parker Physical Sciences Building. The physical facilities for the Center were completed in 1970. Laboratories housing the Leo Goodwin Institute for Cancer Research, a component of the Life Sciences Center, occupy a separate wing and have been especially designed to provide air-handling and isolation facilities adapted to the peculiar requirements of gnotobiology. In addition to a large, glass-walled isolator area, there are separate rooms for preparation of the isolators, steam generators, filters and other special equipment. An isolator research area enables the performance of short-term germfree experiments in a location removed from the main laboratory. The animal cubicles permit the holding of mono-infected or normal animals in isolation.

The opposite wing of the Center contains laboratories for research and teaching in molecular biology, virology, immunology and cell culture. It is provided with large incubator and refrigerator rooms, fume hoods, a photomicroscopy laboratory and sterile transfer cubicles. A Philips EM201 electron microscope was installed in 1972.

Supporting services include a well equipped instrument shop, histology and photomicrography laboratories, refrigerated rooms and incubator rooms for cell culture. In the Reyniers Memorial room is a small collection of books particularly concerned with gnotobiology, and the main library which houses a collection of references in the medical and biological sciences is also located in the Parker Building.

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References

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CURRICULUM VITAE

JOEL WARREN

Birthplace - REDACTED

Married - REDACTED

Education - Yale University, A.B. (Zoology), 1936
Columbia University, A.M. (Bacteriology), 1938
Columbia University, Ph.D., (Bacteriology), 1940

Positions -

REDACTED

Awards - Order Breasted Cloud and Banner, Chinese National Government, 1946
- Meritorious Service Award, U. S. Department of Defense, 1950

Professional Memberships - REDACTED

Publications - Approximately 95 in the general fields of microbiology and infectious diseases

June 1970

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CURRICULUM VITAE

MIRIAM R. SACKSTEDER

Date of Birth: R

Marital Status: R

Education: B.S. Mary Manse College Toledo, Ohio 1949

Positions:

1949-1951

1951-1959

1959-1972

REDACTED

1972-

1971-

Professional Memberships:

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